



Productivity and water use efficiency of *Agave americana* in the first field trial as bioenergy feedstock on arid lands

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Abstract

Agave species are high-yielding crassulacean acid metabolism (CAM) plants, some of which are grown commercially and recognized as potential bioenergy species for dry regions of the world. This study is the first field trial of *Agave* species for bioenergy in the United States, and was established to compare the production of *Agave americana* with the production of *Agave tequilana* and *Agave fourcroydes*, which are produced commercially in Mexico for tequila and fiber. The field trial included four experimental irrigation levels to test the response of biomass production to water inputs. After 3 years, annual production of healthy *A. americana* plants reached 9.3 Mg dry mass ha⁻¹ yr⁻¹ (including pup mass) with 530 mm of annual water inputs, including both rainfall and irrigation. Yields in the most arid conditions tested (300 mm yr⁻¹ water input) were 2.0–4.0 Mg dry mass ha⁻¹ yr⁻¹. *Agave tequilana* and *Agave fourcroydes* were severely damaged by cold in the first winter, and produced maximum yields of only 0.04 Mg ha⁻¹ yr⁻¹ and 0.26 Mg ha⁻¹ yr⁻¹, respectively. The agave snout weevil (*Scyphophorus acupunctatus*) emerged as an important challenge for *A. americana* cropping, killing a greater number of plants in the higher irrigation treatments. Physiological differences in *A. americana* plants across irrigation treatments were most evident in the warmest season, with gas exchange beginning up to 3 h earlier and water use efficiency declining in treatments with the greatest water input (780 mm yr⁻¹ water input). Yields were lower than previous projections for *Agave* species, but results from this study suggest that *A. americana* has potential as a bioenergy crop and would have substantially reduced irrigation requirements relative to conventional crops in the southwestern USA. Challenges for pest management and harvesting must still be addressed before an efficient production system that uses *Agave* can be realized.

Keywords: agriculture, bioethanol, biofuel, CAM, crassulacean acid metabolism, desert, semiarid, southwest USA, WUE

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Introduction

Droughts limit agricultural production in arid and semi-arid regions, yet plants that exhibit crassulacean acid metabolism (CAM) can improve crop yields in these conditions (Cushman *et al.*, 2015; Davis *et al.*, 2015; Yang *et al.*, 2015). Plants in the *Agave* (L.) genus are obligate CAM plants, some of which are produced commercially, and are being considered for bioenergy crops (Borland *et al.*, 2009; Davis *et al.*, 2011, 2014, 2015; Yang *et al.*, 2015). In regions of the world where *Agave* is grown commercially for beverages or fiber, yields range from 8.5 to 22 Mg ha⁻¹ depending on species and location (Davis *et al.*, 2014), but the latitudinal range for these crops is uncertain. Despite the potential advantages and high theoretical yields (Davis *et al.*, 2011,

2014; Owen *et al.*, 2015), there are many dry regions of the world including the southwestern USA where *Agave* has not yet been cultivated commercially or experimentally, making it difficult to assess the realistic potential of this genus for agricultural production. There are no field experiments to date that compare the yields of *Agave* species in arid to semiarid conditions or the response of *Agave* species to variable water inputs. Here, we provide the first field-scale yield estimates of *Agave* in the arid southwestern USA.

Climate projections indicate that many semiarid and arid parts of the world will be subject to more frequent and extreme drought events in the future (IPCC 2014). Agricultural production in many of these areas is already vulnerable to drought, dependent on large volumes of irrigation, and susceptible to soil degradation (Scanlon *et al.*, 2007). The yields of 8.5–22 Mg for *Agave* referenced above (Davis *et al.*, 2014) reflect commercial production in Mexico and Tanzania where the climate

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is semiarid due to prolonged periods of drought even though heavy rainfall does occur intermittently. As water use efficiency (WUE) is the primary advantage of CAM plants that has sparked interest in these plants for dryland agricultural production, there is a need to resolve how much precipitation would be needed to achieve commercially viable yields of *Agave*. Commercial production of bioenergy in the USA is primarily supported by *Zea mays* L. (corn) feedstock, which yields an average of $9.4 \text{ Mg ha}^{-1} \text{ yr}^{-1}$ on prime agricultural land, but the dominant commercial cropping systems in Arizona yield far less (e.g., cotton yields $1.5 \text{ Mg ha}^{-1} \text{ yr}^{-1}$).

The key physiological advantage of CAM, to distinguish from C_3 and C_4 , is the temporal separation of carboxylase activities catalyzed by the enzyme PEPC (phosphoenolpyruvate carboxylase) and the enzyme RUBISCO (ribulose biphosphate [RuBP] carboxylase/oxygenase) so that PEPC is primarily active during the night when stomata are open (opposite of C_3 and C_4 plants), and RUBISCO is primarily active during the day when light energy is available. With PEPC activation occurring at night when temperatures are cooler, evaporative demand is lessened and the ratio of carbon dioxide to water that diffuses through the stomata is increased relative to what would be expected during daytime conditions. The carbon is stored as malic acid overnight in large vacuoles typical of succulent plants and then released by decarboxylation at dawn. This strategy for growth gives CAM plants a strong advantage over C_3 and C_4 plants in conditions of water stress (Osmond, 1978; Nobel, 1996; Winter & Smith, 1996; Davis *et al.*, 2014). Many CAM plants also have thick waxy cuticles, sunken stomata, and roots that are responsive to water stress; all adaptations that hydraulically isolate the plant in extremely dry conditions. Theoretical projections indicate that high-yielding CAM plants such as *Agave* and *Opuntia* (Mill.) can contribute substantially to the global renewable energy portfolio (Davis *et al.*, 2011, 2014; Cushman *et al.*, 2015; Owen *et al.*, 2015).

There is growing interest in *Agave* species for bioenergy production in the arid and semiarid southwestern region of the USA. Commercial agricultural production has been demonstrated for species native to Mexico (e.g., *Agave tequilana* F.A.C. weber and *Agave fourcroydes* Lemair), but these have not been grown in the USA outside of small horticultural and landscaping plantings. *Agave americana* L., also known as the American century plant, is native to the USA (native range includes Arizona, California, Florida, Louisiana, and Texas) and has been reported to reach sizes similar to its high-yielding Mexican relatives (2 m in height before flowering, >3 m in width) and achieve high photosynthetic rates under a

range of environmental conditions (Neales, 1973; Gentry, 1982; Nobel, 2003; Garcia-Moya *et al.*, 2011), but field-scale yields of *A. americana* have not been assessed in previous literature. *A. americana* is produced for a beverage called pulque in Mexico (La Barre, 1938), but there is less information about the agricultural production of this species than what is available from the larger-scale tequila and fiber industries. Tissue composition of *A. americana* has been measured, and the leaves of this species have a low percentage of lignin mass relative to other species of *Agave* as well as high-soluble carbohydrate concentrations relative to other advanced bioenergy crops (Mylsamy & Rajendran, 2010; Li *et al.*, 2012; Corbin *et al.*, 2015), both beneficial traits for biochemical conversion pathways to liquid fuel (Davis *et al.*, 2011).

The first US field trial of *Agave* species for bioenergy was established in 2012 in Maricopa, AZ, a desert agricultural area where annual rainfall averages about 190 mm yr^{-1} . This study reports the results to date for this experiment. The agricultural plantings were designed as a fully factorial two-way experiment to determine differences in survivorship, productivity, and WUE among three species (*A. americana*, *A. tequilana*, and *A. fourcroydes*) and four irrigation treatments (ranging from arid to well-watered conditions), and to determine whether responses to irrigation are species dependent. The study site is at the edge of the climate tolerance range for *A. tequilana* and *A. fourcroydes* (Lewis *et al.*, 2015), but these are the large-scale commercial species against which a comparison of the US native species, *A. americana*, would be meaningful. Significant mortality and cold damage of *A. tequilana* and *A. fourcroydes* occurred after a rare cold event in the first year, so the focus of the study was narrowed by the third year to resolving differences in *A. americana* production, WUE, and survivorship across irrigation treatments. Early survivorship and production of *A. tequilana*, *A. fourcroydes*, and *A. americana* are reported, but differences in carbon dioxide assimilation, biomass yields, and WUE across four irrigation treatments were resolved only for *A. americana* in year 3.

Materials and methods

Study site and experimental design

The study site is a 1.2-ha field located at the University of Arizona Maricopa Agricultural Center (MAC) in Maricopa, AZ, and is centered at $33^{\circ}03'32''$ N latitude, $111^{\circ}58'12''$ W longitude, and 361 m above sea level. The field was plowed, tilled, and leveled before planting. Soil in the field is classified as Casa Grande sandy clay loam, which is described as fine-loamy, mixed, superactive, hyperthermic Typic Natrargids (Post *et al.*, 1988). The field was subdivided into eight sections (each

approximately 32 m by 46 m) separated by berms approximately 50 cm in height to contain water that was allocated to each treatment by way of flood irrigation (Fig. 1). Two replicates of four irrigation treatments were assigned randomly to the eight sections. Irrigation water was provided by a concrete-lined irrigation ditch located on the south end of the field. A 2.5-m long piece of PVC pipe (305 mm, diameter) was hard-plumbed through a hole drilled in the irrigation ditch. Irrigation water to the field was controlled by a swivel valve located inside the ditch at the PVC pipe entry hole. The PVC pipe system was reduced to a 4.6-m long piece of 203-mm-diameter PVC pipe that contained an in-line propeller-driven flow meter. The PVC-valve system was then connected to a 203-mm-diameter plastic polypipe that ran along the center of the field in the north–south direction (Fig. 1). Gates installed along the polypipe at 1.0-m spacing allowed individual sections to be irrigated at desired times. Water was applied in approximately 100-mm increments (the minimum needed to flood-irrigate the section uniformly), but exact amounts varied, as explained below.

Irrigation treatments were initiated in the second year (2013), after planted sections had been uniformly irrigated in 2012 (year one). The control treatment received only precipitation (200 mm annually on average from 2013 to 2014) and the minimal water needed to apply fertilizer. In addition to the native precipitation, the irrigation schedule and levels targeted for other plots were as follows: (i) twice annually (in March and July) to add ~200 mm yr⁻¹ (or ~2 MI ha⁻¹ yr⁻¹), (ii) every other month from March to October to add ~400 mm yr⁻¹ (or ~4 MI ha⁻¹ yr⁻¹), and (iii) monthly from March to October to add ~800 mm yr⁻¹ (or ~8 MI ha⁻¹ yr⁻¹). The actual water inputs achieved differed some from these target quantities due to variations in water flow rate, soil hydraulic and roughness conditions, integrity of berms, which occasionally required

rebuilding after being broken by flood water, and practical decisions made by technicians during sporadic heavy rainfall events. Exact amounts of water applied during irrigation were measured (both flow rate and total volume) by the in-line flow meter. The actual 2-year (2013 and 2014) average amounts of irrigation applied for the four treatment levels were 100 mm yr⁻¹, 260 mm yr⁻¹, 330 mm yr⁻¹, and 580 mm yr⁻¹. Including the mean annual rainfall (~200 mm; see detailed climate in Fig. S1), total water inputs in the treatments were 300 mm yr⁻¹, 460 mm yr⁻¹, 530 mm yr⁻¹, and 780 mm yr⁻¹.

The irrigation treatments described above were initiated in the spring of 2013 (year 2) and repeated through 2014 (year 3). The entire field was fertilized annually in June with N:P:K fertilizer in a ratio of 40:80:80 based on recommendations for commercial plantation management used in the tequila industry. Applied rates each year were 56 kg N ha⁻¹, 112 kg P ha⁻¹, and 112 kg K ha⁻¹. Weeding was accomplished manually in the first year, and required regular attention (twice per month). In the second and third years, weeds were controlled with herbicide (Surflan) applied 3–4 time per year at a rate of 4.7–9.5 l ha⁻¹, by mowing between rows where possible, and manual hoeing of weeds near the base of plants.

Within each of the replicated irrigation sections, there were six plots of *Agave* for a total of 48 plots across the field (Fig. 1). Two plots each of *A. tequilana*, *A. fourcroydes*, and *A. americana* were randomly assigned within each replicated irrigation treatment, so that across the whole field there were four replicate plots of each species in each irrigation treatment. Each plot was an experimental unit and contained 49 plants (7 × 7) spaced 2 m apart both within and along rows. The experimental design in the field was purposeful to statistically test the difference in survivorship, yield, and WUE among species and among irrigation treatments. We treated this as a fully factorial design instead of a split-plot design because each combination

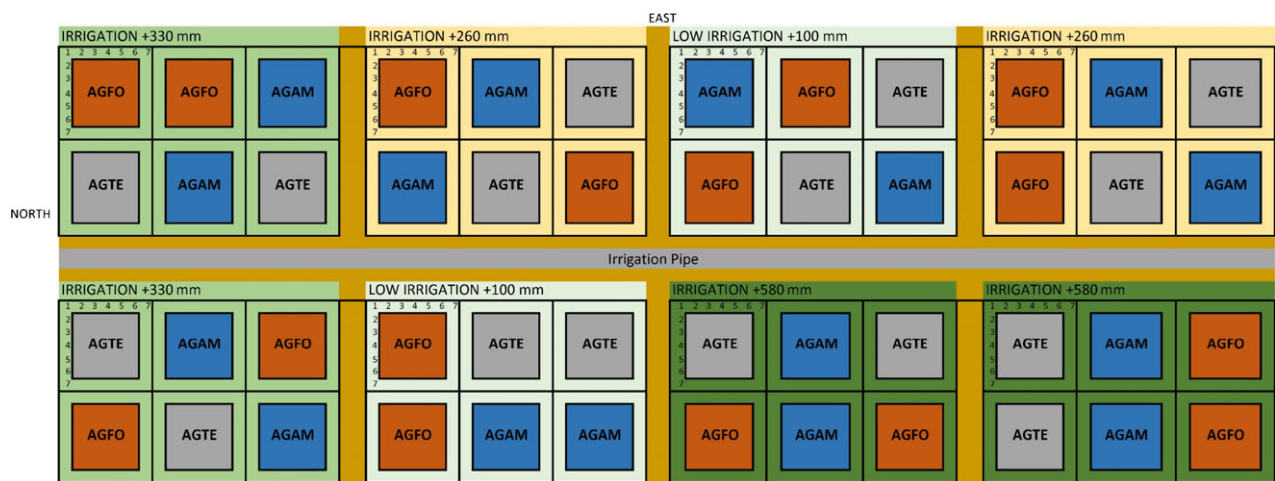


Fig. 1 Field layout of the experimental design with three species (AGAM: *A. americana*, AGFO: *A. fourcroydes*, and AGTE: *A. tequilana*) and four irrigation treatments. Each square plot contained 49 plants spaced 2 m apart. Four replicate plots of each species were randomly assigned to each irrigation treatment with two replicated and randomly located areas of the field assigned to each irrigation level (100, 260, 330, and 580 mm yr⁻¹). Total water inputs including rainfall for each treatment were 300, 460, 530, and 780 mm yr⁻¹. The irrigation pipe (shown in gray) was laid through the center of the field to allow access to each section that would be flood-irrigated at separate times. Berms (shown in brown) were constructed to separate each irrigated section.

of irrigation and species treatments was replicated throughout a single field and the sections delineated in the field for each irrigation were not unique.

Agave tequilana plants were a single genotype of weber var. azul propagated clonally and purchased from growers in Oaxaca, Mexico. *Agave fourcroydes* plants were mixed unidentified genotypes purchased from growers in Yucatan, Mexico. Both *A. tequilana* and *A. fourcroydes* were imported bare rooted with permits from the U.S. Department of Agriculture and were uniform in age of just under 1 year. *Agave americana* plants were from a variety of unidentified genotypes purchased from a grower in California, USA, and shipped to the site in one gallon pots (also approximately 1 year in age).

All species were planted in the spring of 2012 between March 23 and May 5 and irrigated weekly for the first month to ensure establishment, then irrigated monthly until October 2012 (~1000 mm water applied in total). Establishment was successful for all three species, and there was no mortality observed prior to January of 2013. There was an unusually cold period with nine consecutive days of temperatures below freezing in January of 2013, and minimums between -6°C and -8°C for five consecutive nights. The *A. tequilana* and *A. fourcroydes* plots suffered severe tissue damage. The monthly mean minimum temperature was 0.6°C , 67% lower than the mean minimum for January in the other growth years. Although many plants remained, they were severely damaged and the biomass of both *A. fourcroydes* and *A. tequilana* were magnitudes lower than the biomass of *A. americana* as a result. The study is therefore focused on the response of *A. americana* to irrigation, although survivorship and yields were quantified and compared among species after year two. There were an insufficient number of plots of *A. tequilana* remaining across irrigation treatments to statistically assess the response to irrigation for this species.

Measurements

A field-calibrated neutron probe (Model 503, Campbell Pacific Nuclear, CPN, Martinez, CA) was used to measure the volumetric soil water contents (θ) of plots from 0.1 m to 1.9 m in 0.2 m incremental depths. Between mid-April and mid-May, 2013, 33 neutron access tubes were installed vertically to a soil depth of 2.0 m using a tractor-mounted Giddings soil sampler (Model 25-TS; Giddings Machine Company, Windsor, CO, USA). Of the 33 tubes, 16 were located within each of the 16 *A. americana* plots (i.e., four per irrigation level). Tubes were installed at a distance of about 1.0 m away from plants. During installation of the neutron access tubes, soil samples in 0.3-m increments were collected at each location to a depth of 1.8 m (with 6 depth increments). Each separate soil sample was analyzed for soil particle size fraction using the Bouyoucos hydrometer method (Gee & Bauder, 1986). Although sandy clay loam and sandy loam textures were predominate, soil texture analyses revealed approximately 10% of the total samples were classified either as clay loam, loam, or sandy clay. The volumetric soil water content measurements at the 33 access tube locations began on May 21, 2013. In general, soil water contents were measured twice monthly, just before and a few

days after a scheduled irrigation. Soil water contents were measured for all access tubes and depths with each irrigation event, even when only a subset of the plots were irrigated.

Evapotranspiration (ET_c) for the irrigation treatments of *A. americana* was determined over successive soil water content measurement dates as the residual of the soil water balance of an estimated root zone of 2.0 m. Following Jensen *et al.* (1990), the soil water balance for ET_c occurring between the first measurement date, denoted as day $i = 1$, and the second date ≈ 15 days later on day n was calculated as:

$$\text{ET}_c = \sum_{i=1}^{10} (S_{i,1} - S_{i,n}) + \sum_{j=1}^{n-1} (R_j + \text{IW}_j) \quad (1)$$

where ET_c is the total evapotranspiration that occurred from day 1 to day n , $S_{i,1}$ and $S_{i,n}$ are soil water storage measured at each of the 10 neutron probe soil depth layers on day $i = 1$ and on day n , respectively. R_j and IW_j are, respectively, the measured rainfall and applied irrigation depths received on day j . All units are in mm. Rainfall data were provided by a University of Arizona, Meteorological Network (AzMet; ag.arizona.edu/azmet) weather station located at MAC, about 1.2 km from the field site. For each of the 10 soil depth increments, soil water storage was calculated as the measured soil water contents (m^3/m^3) at the layer times the soil depth layer in m (i.e., $\theta \times 0.2$ m). For each soil water measurement location, the volumetric soil water contents at field capacity (FC) and permanent wilting point (PWP) were determined for each layer from the soil texture data using soil water characteristics estimation procedures developed by Saxton & Rawls (2006). Equation 1 does not include a deep percolation component, as there was no evidence of soil water content increasing below a soil depth of 1.5 m. Fig. 2 shows the periodically measured θ for each layer with time for the wettest irrigation treatment (i.e., 580 mm). The figure includes the FC and PWP for each layer determined from soil texture. For 2013 (Fig. 2a), soil water contents measured a few days after irrigations increased to a depth of 1.5 m. However, the soil water contents at lower layers did not increase above FC, indicating negligible drainage from the root zone. Conversely, it can be seen, particularly in 2014 (Fig. 2b), that soil water was depleted somewhat to 1.9 m, suggesting that a root zone of 2.0 m for agave water use was reasonable.

Survival and reproduction rates of plants were recorded and compared across species and irrigation treatments in the second year of growth. Survival rates were recorded in July of 2013 (after all cold damage could be assessed) and biomass productivity was recorded in January of 2014 (after two full growing seasons). The mean percentage of surviving plants in four replicate plots was used to estimate survivorship of each species in each irrigation treatment. No significant cold damage was observed after the spring of 2013. Survivorship and biomass productivity was measured again in February of 2015 (after three full growing seasons). The mean number and condition of reproductive offsets (pups) was also measured in each plot.

The response of dusk-to-dawn carbon dioxide uptake to the different irrigation treatments was determined by measuring gas exchange in *A. americana* using Li-Cor LI-6400XT Portable Photosynthesis Systems (Li-Cor; Lincoln, NE, USA). These infrared gas analyzers were used to record carbon uptake rates

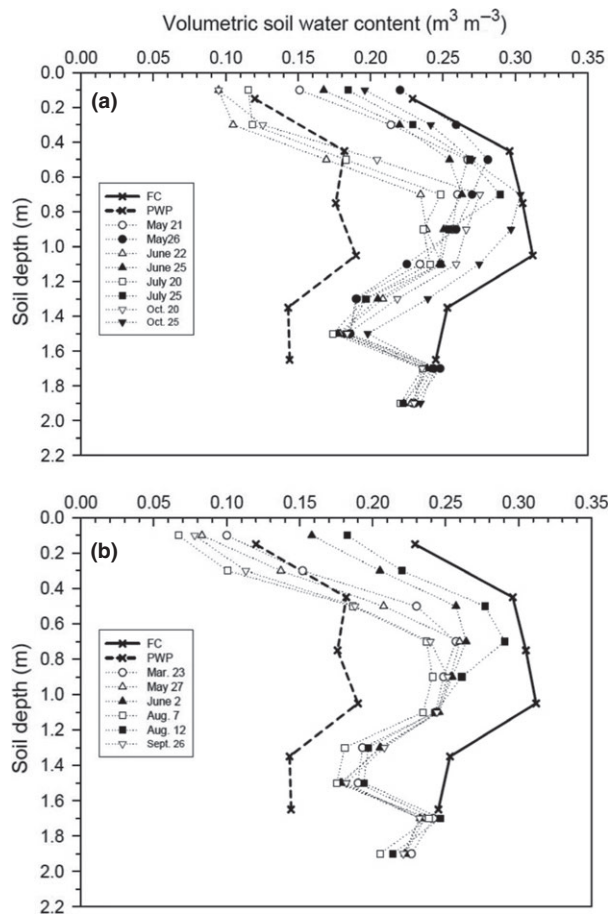


Fig. 2 Soil water content measurements from 0.1 to 1.9 m, in 0.2-m increments (dotted lines), field capacity (FC, solid line), and permanent wilting point (PWP, dashed line) derived from soil texture analyses for 0.3-m soil layers from 0 to 1.8 m for the 800-mm treatment plot in 2013 (a) and 2014 (b). Only a subset of all measurements are shown as examples. Note soil water content measurements made after an irrigation are denoted by dark symbols. The FC and PWP data are plotted at the center of the layer depth (e.g., at 0.15 m for the 0- to 30-m layer).

for plants in each irrigation treatment over a time course starting before sunset and extending until after dawn at three times throughout the year (March, June, and November). Total net carbon assimilation was estimated by integrating the measurements of gas exchange over time (typically a 15-h period). Sampling began in the late afternoon in attempt to include gas exchange during Phase IV, but gas exchange was not observed prior to 6 pm. In November of 2014, a full 24-h time period was measured to verify that the 15-h measurements reflected the full duration of stomatal opening. Dynamics of gas exchange were also compared to determine whether the timing of peak carbon uptake rates and the phases of CAM differed across the four different irrigation treatments.

During each seasonal sampling, gas exchange over the 15-h period from 6:00 pm to 9:00 am was measured at approximately 30-min intervals in each irrigation treatment. Each incremental estimate during the diurnal time period was a

mean of the gas exchange rate measured in a mature leaf located midway between the outside whorl and center spike of three randomly selected plants in each irrigation treatment. This approach allowed us to estimate gas exchange levels using a mean of several plants within an irrigation treatment instead of that of a single plant. A square 2 cm × 3 cm cuvette was used with thick gaskets to accommodate the thick succulent leaves, and measurements were made at a point on the leaf approximately two thirds the distance from the base. There were occasional gaps in data (a missing 30-min increment) due to the need to switch batteries in the LiCor 6400 or other maintenance requirements. When calculating the total dusk-to-dawn CO₂ assimilation, measurements from the next closest time interval were used to estimate fluxes during each gap.

Aboveground biomass of a random plant from each of the sixteen *A. americana* plots was measured after destructively harvesting at the end of second and third years of growth (mid-January 2014 and early February 2015). In February of 2015, aboveground biomass of all plants that were killed by the agave snout weevil was also measured (the number of plants killed per plot ranged from 3 to 38). Because most plants harvested in February of 2015 were infested with the snout weevil, an additional harvest occurred in the spring of 2015 to assess the productivity of plants that remained healthy. Subsamples from each plant, consisting of three randomly selected leaves, were dried in an oven for 2 weeks at 65 °C and then weighed to estimate plant tissue water content. The water mass was subtracted from the total wet biomass to estimate dry biomass production in each plot.

Net WUE was estimated in two ways: WUE₁, as total dry biomass per unit of water input (including both precipitation and irrigation); and WUE₂, as total dry biomass per unit ET_c, as calculated from the soil water balance described above. WUE₁ provides a practical estimate for growers of the amount of biomass one can expect given a certain water input. WUE₂ can be used to resolve any additional soil water that might be used to supplement the plant water requirements.

The statistical program JMP Pro 10 (SAS Institute Inc., Cary, NC, USA) was used to assess the differences in survivorship and biomass productivity in year 2 among species and irrigation treatments, and whether the effect of irrigation depended on species, using a two-way analysis of variance (ANOVA). The same statistical program was used to test differences in (i) survivorship, (ii) reproductive offsets, (iii) biomass productivity, and (iv) WUE of *A. americana* among irrigation treatments in year 3 using a one-way ANOVA. The Shapiro-Wilk W-test was used to determine whether the distribution in response variables met the assumption of normality. Tukey's HSD (honest significant differences) was used to resolve significant differences among treatment levels. Linear regression was used to compare the two estimates of WUE, and nonlinear regression was used to assess the response of WUE to increasing water inputs and ET_c.

Results

Initial establishment of *A. americana*, *A. tequilana*, and *A. fourcroydes* was successful, with a mean survival at

the plot level of 100%, 97%, and 92%, respectively, by August of 2012. The few plants that were lost during this period were replaced. In January of 2013, the minimum temperature on five consecutive nights ranged from -6°C to -8°C , and cold damage was immediately evident in the *A. tequilana* and *A. fourcroydes* even though mortality was not entirely evident until March of 2013. A survey of the field in July of 2013 revealed that 74% of the *A. tequilana* plants were killed by the prolonged cold (Fig. 3), and 88% of the *A. fourcroydes* were severely damaged. There was no mortality or cold damage observed in the plots of *A. americana* after the cold event in January of 2013, and survival of the *A. americana* remained near 100% until 2014. There was greater mortality in *A. tequilana* than in *A. fourcroydes* or *A. americana*, and irrigation treatments did not affect survival in the second year for any species. Statistical significance is not reported for these differences because the distribution of response variables was bimodal (violating the assumption of normality in the statistical model) and could not be corrected through transformation. Ranking data for analysis using nonparametric tests was not possible because survival was 100% for all *A. americana* plots (insufficient range for ranking). Nevertheless, the difference in survivorship among species was clear (Fig. 3).

Although many of the *A. fourcroydes* survived, the cold damage was severe and the aboveground tissue completely died back in many of the plots. After recovery, the aboveground biomass of *A. fourcroydes* in year two was less than $0.3\text{ Mg ha}^{-1}\text{ yr}^{-1}$, even in the highest irrigation treatment, and was less than 9% of the biomass production measured for *A. americana* in all irrigation treatments (Fig. 4). While there was a significant main effect of irrigation on biomass ($P < 0.01$), this response differed by species ($P < 0.01$), with *A. americana* biomass increasing more with irrigation levels than biomass of *A. fourcroydes*. *A. americana* biomass was also significantly greater than *A. fourcroydes* biomass in all irrigation treatments ($P < 0.01$). Before statistically resolving the differences between *A. americana* and *A. fourcroydes*, biomass data were log-transformed to achieve a normal distribution. There were so few *A. tequilana* surviving that replicate plots of living plants no longer remained in all treatments (some plots had 0 surviving plants), but the biomass production of the remaining plots was $\leq 1\%$ of *A. americana* production (Fig. 4). *A. tequilana* and *A. fourcroydes* did not recover nor make any significant gain in biomass production by the end of the third year.

The first evidence of mortality in the *A. americana* plots was recorded in January of 2014, and this was due to an infestation by *Scyphophorus acupunctatus* (agave snout weevil). The plots were first treated with an

insecticide (Merit, Bayer CropScience, Monheim, Germany) in February of 2014, and two repeat applications occurred in the summer and fall (each at a rate of $67\text{--}87\text{ kg ha}^{-1}$). The weevil was, however, never fully eradicated from the field. In February of 2015, 77% of the originally established plants (mother plants) in the highest irrigation treatment had been killed by the snout weevil, significantly more than the other irrigation treatments ($P < 0.01$). Survivorship was greatest (80%) in the plots with the lowest annual water inputs (Fig. 5).

Asexual reproduction rates of *A. americana* were greater in the plots with 780 mm annual water inputs in 2014, but the mean number of reproductive offsets (pups) produced for each original plant (mother plant) was similar across treatments in 2015 (Fig. 6). An average of 3 pups per mother plant were produced across

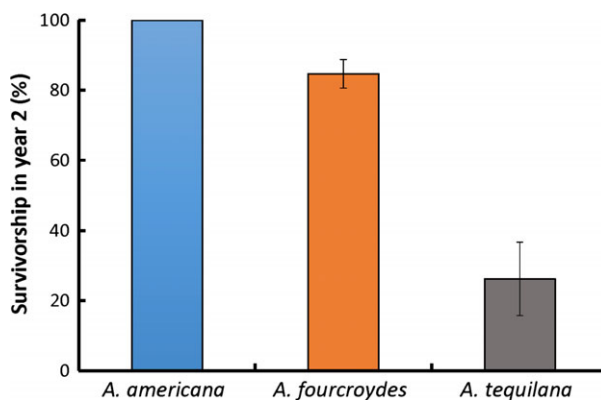


Fig. 3 Percent survivorship of three *Agave* species by July of 2013. Bars represent means of replicate plots across all irrigation levels ($n = 16$), and error bars represent standard error.

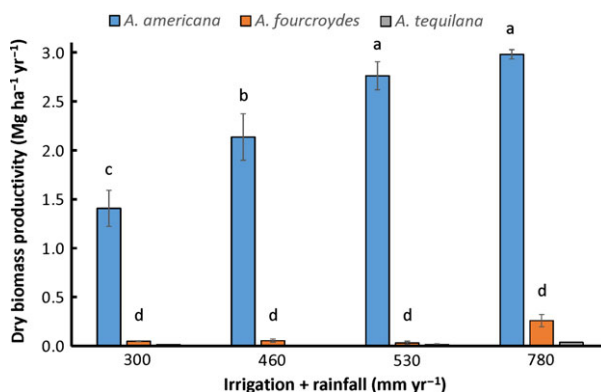


Fig. 4 Annualized productivity of three *Agave* species across the four irrigation treatments by January of 2014. Bars represent treatment means with plots as the replicate ($n = 4$), and error bars represent standard error. Different letters indicate statistically significant differences ($P < 0.05$).

all plots of *A. americana* in 2014, and a mean of 4 pups per mother plant were produced by 2015. There was not a significant difference among irrigation treatments in the number of surviving pups remaining in the plots ($P = 0.14$), or in the total number of pups produced per mother plant ($P = 0.10$), although the trend in 2015 was toward greater pup production with annual water inputs of 460 mm yr^{-1} and 530 mm yr^{-1} . Reproductive offsets were not observed in the other species.

Biomass of *A. americana* plants increased with water inputs of up to 530 mm in all years (Fig. 7). In 2014, plots with the greatest water inputs (530 mm yr^{-1} and 780 mm yr^{-1}) resulted in the highest annual biomass

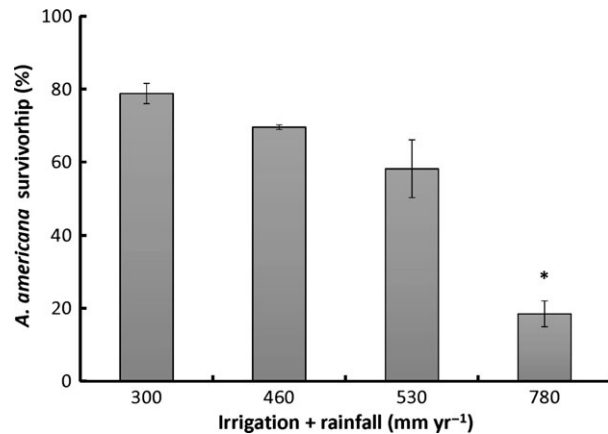


Fig. 5 Percent survivorship of *A. americana* after the agave snout weevil spread through the field in 2014. Survivorship was assessed in February of 2015. Bars represent treatment means with plots as the replicate ($n = 4$) and error bars represent standard error. * indicates statistically significant difference ($P < 0.05$).

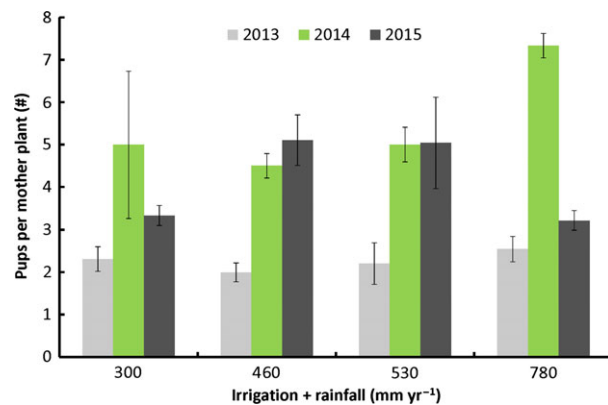


Fig. 6 Number of asexually propagated offsets (pups) observed for each *A. americana* plant that was initially established in the field (mother plants). Bars represent mean of replicate plots ($n = 4$), and error bars represent standard error.

production (2.8–3.0 $\text{Mg ha}^{-1} \text{yr}^{-1}$), significantly greater than the 300-mm or 460-mm treatments ($P < 0.01$). The difference among treatments in biomass of dead plants harvested in 2015 was not significant ($P = 0.77$). There was, however, a trend for greater biomass productivity of healthy mother plants in the plots that received 530 mm yr^{-1} of water when compared to those in the other treatments ($P = 0.06$; Fig. 7). Total aboveground biomass, including biomass of both pups and mother plants, was significantly different across irrigation treatments in 2015 ($P = 0.02$), with the biomass production reaching 9.3 $\text{Mg ha}^{-1} \text{yr}^{-1}$ in plots with 530 mm of annual water input (Fig. 7). After 3 years, annual production of healthy plants was more than double the annual production after 2 years, although mean production rates of plants killed by the snout weevil were much lower (Fig. 7).

WUE₁, calculated as total aboveground biomass production per unit of total water input, was similar in irrigation treatments that ranged from total inputs of

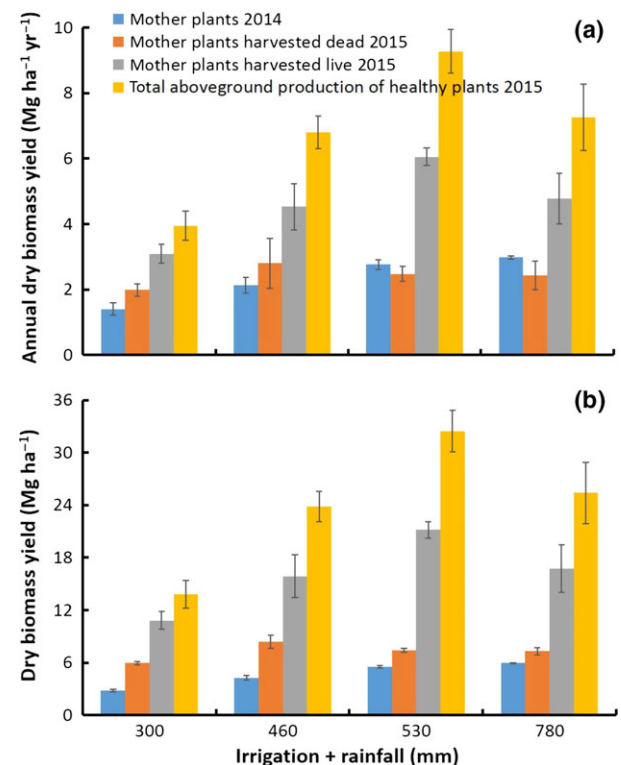


Fig. 7 *Agave americana* oven-dried biomass production annualized (a) and total (b) for 2-year-old mother plants (blue, 2014), dead 3-year-old mother plants (orange, 2015), living 3-year-old mother plants (gray, 2015), and total aboveground biomass of healthy mother and pups (yellow, 2015). Bars represent a mean of individuals from replicate plots ($n = 4$), but the number of dead plants per plot varied ($3 < n < 31$) and error bars represent standard error.

300 mm yr⁻¹ to 530 mm yr⁻¹ ($P > 0.05$), but WUE_1 declined in the highest irrigation treatment and was significantly lower with inputs of 780 mm yr⁻¹ ($P = 0.01$; Fig. 8a). Trends in the response of WUE_2 , calculated as total aboveground biomass production per unit of ET_c (Fig. 8b), to water inputs were very similar to the response of WUE_1 because there was little ET_c derived from soil water storage (Table 1). Results from the two methods estimating WUE were closely correlated (Fig. 8d; $R^2 = 0.99$). There was a nonlinear correlation between water inputs and WUE_1 ($R^2 = 0.88$) and between ET_c and WUE_2 ($R^2 = 0.82$), with the greatest WUE resulting from conditions with total water inputs of 460 mm yr⁻¹ to 530 mm yr⁻¹ (Fig. 8).

The greatest difference in carbon assimilation among treatments, as measured by dusk-to-dawn gas exchange, occurred in June (Fig. 9), which was the time period

with the lowest native rainfall (relative to March and November). During the dry summer nights, plants growing in plots with higher water inputs (530 mm and 780 mm) opened stomata up to 3 h earlier than plants growing in the control plots (Fig. 10). Carbon assimilation increased with irrigation treatment levels in June, but differences across treatments were not evident in March and November (Fig. 9). The measurement campaign in November confirmed that there was no mid-day carbon assimilation.

Discussion

Agave americana was successfully established as an agricultural planting in the arid conditions of the southwestern USA. Although biomass production of *A. americana* responded to irrigation, there was no bene-

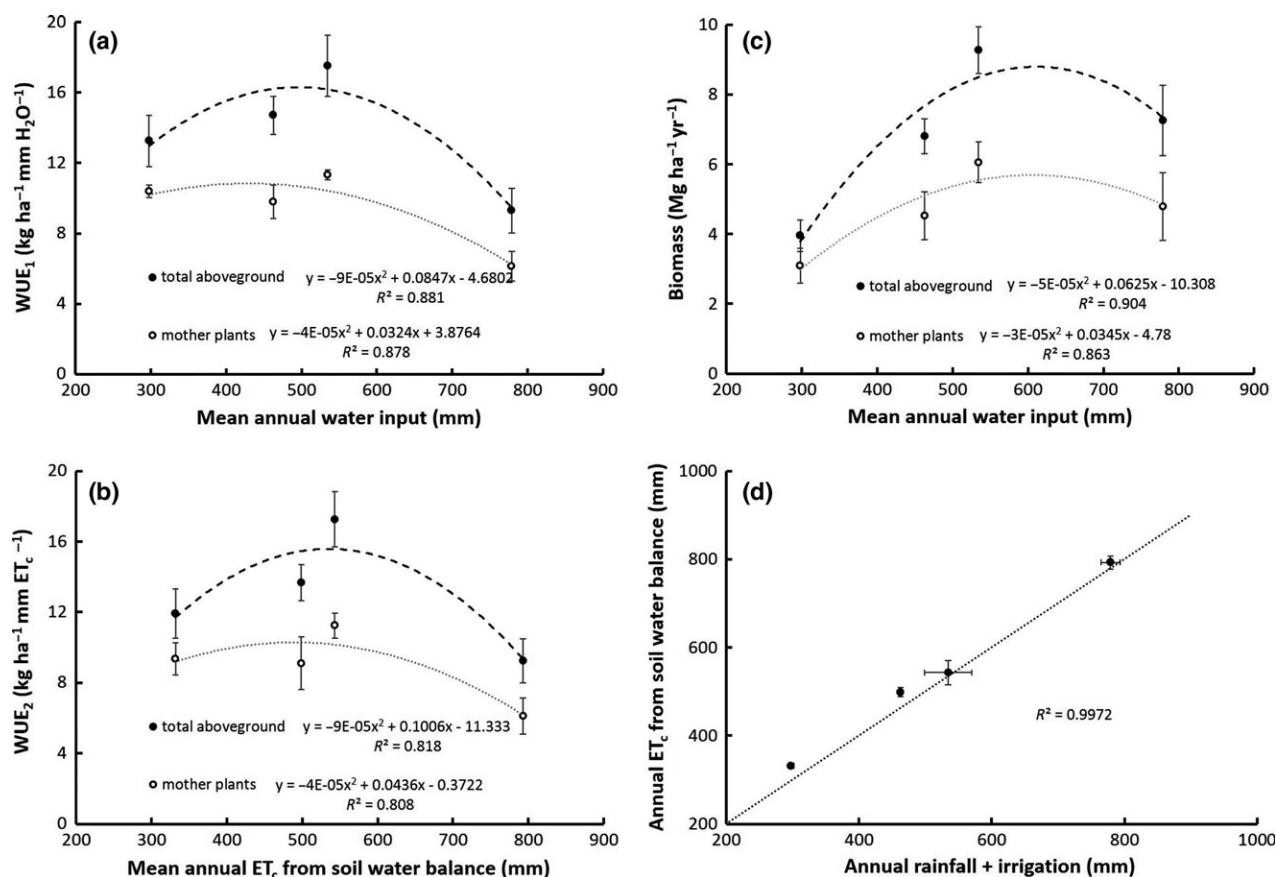


Fig. 8 Relationships between increasing annual water inputs and water use efficiency calculated as the biomass per unit of water inputs, WUE_1 (a); between increasing annual crop evapotranspiration calculated from soil water balances, ET_c , and water use efficiency calculated as biomass per unit of ET_c , WUE_2 (b); and between increasing annual water inputs and oven-dried biomass (c) of *A. americana*. Each point in panels (a), (b), and (c) represents a mean of individuals from replicate plots ($n = 4$), open circles indicate biomass or WUE of mother plants only, and closed circles indicate biomass or WUE of all aboveground plant mass including mother plants and pups. Data here are representing only healthy living plants. Also shown is the close relationship between annual water input (rainfall + precipitation) and annual ET_c (d). Each point in panel (d) represents a mean of replicate irrigation sections in the experimental field ($N = 2$). All error bars indicate standard error.

Table 1 Treatment means* for *Agave americana* soil water balance components† expressed as yearly averages for 2013–2014, where IW is exact irrigation applied without rounding, R is measured rainfall, ΔS ‡ is the change in soil water storage within a soil profile of 2.0 m, and ET_c is annual crop evapotranspiration

Treatment	IW (mm)	R (mm)	ΔS (mm)	ET_c (mm)
580	577	202	14	793
330	333	202	8	543
260	261	202	36	499
100	96	202	34	332

*Treatments are denoted as amount of annual irrigation applied.

†Note deep percolation water penetrating below 2.0 m was assumed negligible for all treatments.

‡ ΔS is the change averaged from Jan. 1 to Dec. 31 in 2013 and from Jan. 1 to Dec. 31 in 2014.

fit of irrigating beyond 330 mm yr⁻¹ and the optimum water input (including rainfall) for an *A. americana* crop was ~530 mm yr⁻¹. Yields under these conditions ranged from 2.5 to 9.3 Mg ha⁻¹ yr⁻¹ (dry mass) over 3 years depending on pest infestations and whether harvests included only mother plants or all plant biomass. The lower production estimate is comparable to that of soybean (~2.9 Mg ha⁻¹ yr⁻¹), a crop grown for biodiesel, and the upper production estimate is comparable to corn grain (~9.4 Mg ha⁻¹ yr⁻¹), a feedstock for bioethanol production and the most abundant biofuel crop globally, in prime agricultural regions of the USA based on the national average of these crops over the last 5 years (NASS, 2015).

In the most arid conditions tested here (300 mm of annual water inputs), biomass production of *A. americana* ranged from 2.0 to 4.0 Mg ha⁻¹ yr⁻¹, indicating that *A. americana* crops may have commercially viable yields in dry regions with minimal irrigation required for establishment. Approximately 30% of global land is arid or semiarid, most of which is considered poorly suited or unsuitable for agriculture (van Velthuisen *et al.*, 2007), and *Agave* crops offer opportunity for production in these regions. Irrigation for agriculture consumes more water than any other practice and continued high consumption is unsustainable, especially in dry regions (Giovannucci *et al.*, 2012).

In the southwestern USA, agriculture that relies heavily on irrigation is economically unstable. Cotton, for example, one of the most important crops in Arizona, requires an average of 1,046 mm yr⁻¹ of irrigation water (in addition to rainfall) and yields only 1.46 Mg ha⁻¹ annually (USDA 2012). This high water demand is increasingly problematic; recent estimates suggest that 20–60 Mha of irrigated cropland globally

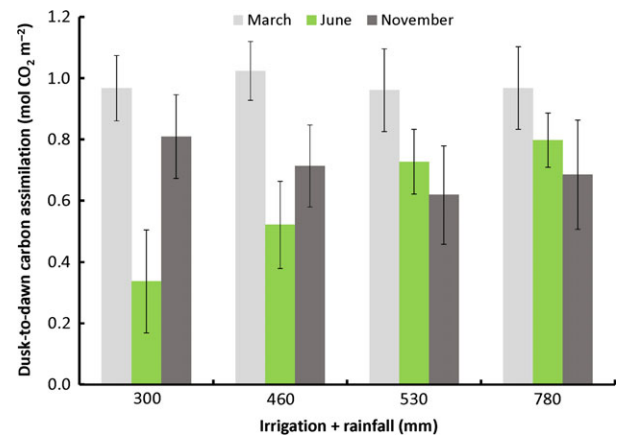


Fig. 9 Net CO₂ uptake of *A. americana* measured at approximately 30-min intervals between the hours of 6 pm and 9 am in March (light gray), June (green), and November (dark gray) of 2014. Each bar represents a mean of individuals ($n = 3$ for most time points) in each irrigation treatment, and error bars represent the standard error integrated over the full 15 h sampling period. Note that 29 mm of rain occurred prior to the March measurements, soil conditions were dry during the June measurements, and the agave snout weevil was disproportionately present in the higher irrigation treatments in November.

will be forced out of production by the end of the century due to a decline in the availability of freshwater resources (Elliott *et al.*, 2014). *A. americana* is an alternative crop that can be cultivated in dry climates with as little as a tenth of the water inputs required from conventional crops.

Although moderately productive with 300 mm – 460 mm of annual water input, the soil water balance measurements (Table 1) suggest that a greater amount of stored soil water was withdrawn by *A. americana* in these conditions (Fig. 8d). The additional water demand is small (34–36 mm), but could have an impact on soil water recharge rates in dry regions if commercially scaled plantations were established. Effects of agriculture on groundwater recharge are a major challenge for the southwestern USA (*e.g.*, Scanlon *et al.*, 2005). The optimum annual water input therefore seems to be between 460 mm and 530 mm of total water inputs. At inputs ≥ 530 mm, ET_c is nearly equal to water inputs (Fig. 8d).

The agave snout weevil is a major threat to crops of *A. americana*, and the impact of the snout weevil on *A. americana* survival increased with irrigation levels. The susceptibility of the plants in the high irrigation treatments could be due to the larger initial size of these plants. Horticultural texts indicate that the weevils preferentially burrow into larger plants to reproduce (Irish & Irish, 2000). Alternatively, and because the plant mortality due to the snout weevil was observed in plants

that were only 2 years old, the additional moisture in the high irrigation treatments may provide an optimum environment for the agave snout weevil.

The agave snout weevil is a well-known threat to *A. americana* plants, but effective treatments have not been fully developed. Recent studies indicate that extracts from castor oil plants (*Ricinus communis* L.) may be effective repellents for adult weevils (Pacheco-Sánchez *et al.*, 2012a,b). Adult weevils burrow into the stem of the *Agave* plant and reproduce there, so the larvae are nourished by the readily available carbohydrates at the leaf bases. The result is that the base of the stem is destroyed before the rest of plant shows symptoms (Fig. 11). The agave snout weevil is widespread in the United States, and should be expected in an *A. americana* crop unless a resistant genotype is identified. There is some anecdotal evidence of resistant genotypes (Irish & Irish, 2000), and it is possible that resistant individuals will be identified as this field trial continues.

If the timing of the snout weevil infestation can be delayed until at least the third or fourth growing season, which seems most likely in conditions with low

water inputs, biomass yields can still be competitive with other bioenergy crops. It is noteworthy that the plants are easier to harvest manually after the snout weevil has weakened the stems, but minimizing the spread of this pest would be most desirable. Commercial production in the USA will likely require mechanized harvesting instead of the traditional manual harvesting that is common in Mexico. A recent economic analysis indicated that manual harvests were too costly for economically viable production of *Agave* for biofuel in the USA (Nuñez *et al.*, 2011). Existing mechanized harvesters could be modified to cut the plants at the base as in tequila plantations or to trim leaves annually as in fiber plantation.

This study is the first field experiment to compare *Agave* production under a range of controlled water inputs. The production of *Agave* species measured in this study was lower than previously reported *Agave* biomass in semiarid regions (e.g., Davis *et al.*, 2011), but *A. tequilana* production was similar to modeled projections of *A. tequilana* biomass potential in the United States (Owen *et al.*, 2015). There are no previous reports

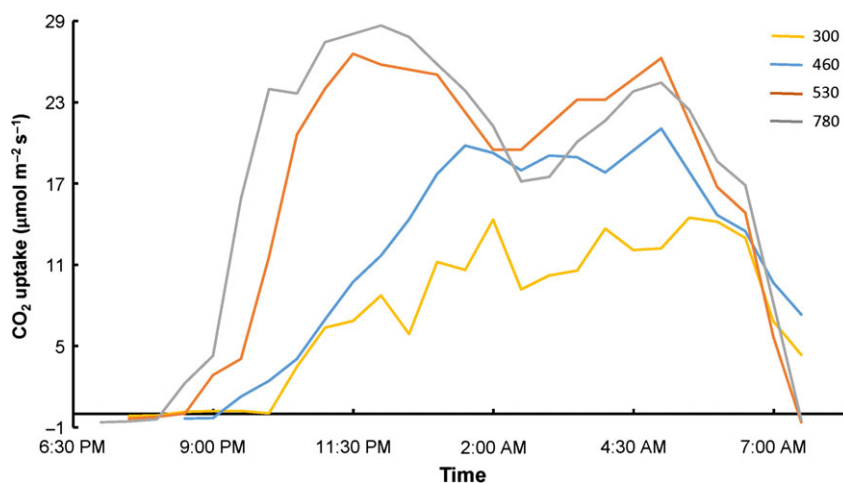


Fig. 10 Dusk-to-dawn CO₂ uptake of *A. americana* measured in June of 2014 with four levels of water inputs (290 mm yr⁻¹, yellow; 410 mm yr⁻¹, blue; 520 mm yr⁻¹, orange; 680 mm yr⁻¹, gray).



Fig. 11 Stem of *A. americana* affected by the agave snout weevil (left) and the stem of a healthy *A. americana* (right).

of *A. americana* production, but yields were lower than theoretical production rates for CAM plants (Nobel, 1991; Borland *et al.*, 2009; Davis *et al.*, 2014), and far below the theoretical predictions of 43 Mg ha⁻¹ yr⁻¹ for high-yielding CAM plants (Borland *et al.*, 2009). Davis *et al.* (2014) projected that, in arid conditions with only 200 mm annual precipitation, optimum yields of CAM plants could be 8.9 Mg ha⁻¹ annually. Although the lowest irrigation treatment in this study, with 300 mm total water input, yielded 2.0–4.0 Mg ha⁻¹ yr⁻¹, it is possible that a more mature crop (≥4 years), optimized genetic lines of *A. americana*, or other cold-tolerant *Agave* spp. will yield greater biomass.

Changes in planting density can have substantial impact on biomass production per unit area. The planting density of each plot in this study was ~2500 plants/ha. This is consistent with plant spacing used in sisal production and tequila plantations, and allows enough space for field managers to move between rows. If planting density could be increased to 4000 plants/ha, and managed with specialized equipment or remote technology, then higher yields could be expected. The crop canopy was not closed (there was exposed ground) in all plots measured in this study.

The results of this experiment to date indicate that *A. tequilana* and *A. fourcroydes*, two important commercial species in Mexico, were not productive 3 years after planting in Maricopa, AZ. This region has been predicted to have marginally appropriate growing conditions for *A. tequilana*, while more optimal conditions are predicted in Texas, California, and Florida (Lewis *et al.*, 2015; Owen *et al.*, 2015). Still, the sensitivity of these species to cold make them vulnerable to sporadic extreme climate events, and additional risk would be introduced when planting these species in the southwestern USA. Variable cold tolerances among *Agave* spp. have been observed and described in previous literature (*e.g.*, Nobel & Smith, 1983). The response of the tonoplast to temperature increases during the day has been implicated in controlling phase changes that are essential to CAM (Kluge & Schomburg, 1996), but research to resolve mechanisms of cold tolerance in CAM plants is needed. Carbohydrate concentrations, particularly high molecular weight fructans, are correlated with freeze tolerance in grasses (Dionne *et al.*, 2010), and may explain the difference in cold tolerance between *Agave* species in this study because *A. tequilana* leaves have lower concentrations of water-soluble carbohydrates than *A. americana* leaves (Li *et al.*, 2012; Corbin *et al.*, 2015).

Past life-cycle assessment indicated that there was potential for *Agave* to have equal or greater environmental benefits for displacing fossil fuels when compared to sugarcane (*Saccharum officinarum* L.), corn (*Zea mays* L.), and switchgrass (*Panicum virgatum* L.) (Yan *et al.*, 2011).

Yan *et al.* (2011) assumed production rates of approximately 30 Mg ha⁻¹ after 6 years (5 Mg ha⁻¹ yr⁻¹) in *A. tequilana* stems, the sugar content of which was assumed to be 80% of the dry mass. Corbin *et al.* (2015) recently estimated the sugar content of *A. americana* plant tissue to be 60% of dry mass, suggesting that bioenergy production from *A. americana* in the southwestern USA could have similar life-cycle benefits if the optimum yields (6.1–9.3 Mg ha⁻¹ yr⁻¹) observed in this study were achieved. The feasibility of a full commercial production system that uses *A. americana* as feedstock depends on other economic factors specific to the location where it would be cultivated.

Conclusions

Agave americana is a potential alternative bioenergy crop for the southwestern USA, even in arid conditions. *A. tequilana* and *A. fourcroydes* were vulnerable to extreme cold events that can occur in this region, and were far less productive than *A. americana* in this side-by-side trial. The agave snout weevil poses a clear risk to the production of *A. americana*, but it may be manageable in conditions where annual water inputs are ≤530 mm. Genotypic selection for resistance to the agave snout weevil might also reduce this problem in the future. Healthy 3-year-old *A. americana* plots yielded 4.0–9.3 Mg ha⁻¹ yr⁻¹ total biomass with 300–530 mm yr⁻¹ of water input (including 100–330 mm yr⁻¹ irrigation, respectively), and plot yields without pup mass included were 3.1–6.1 Mg ha⁻¹ yr⁻¹. The production rates of *A. americana* were greater than conventional crops in Arizona (*e.g.*, cotton) with less water input than is typical in this dry agricultural region.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Monthly mean temperature (top) and precipitation (bottom) patterns during the study period.